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Hybrid of Monkey and Human Adenoviruses

A. D. ALTSTEIN, N. N. DODONOVA, N. N. VASSILYEVA, AND E. M. TSETLIN

L. A. Tarassevich State Control Institute for Medical Biological Preparations, Moscow, USSR

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Following joint replication of monkey SA7 adenovirus (C8 strain) and human adenovirus type 2 in green monkey kidney tissue culture, a virus possessing the properties of a hybrid was obtained. It was designated Ad2C8. Ad2C8 preparations contained two types of viral particles: human adenovirus type 2, and hybrid particles. The hybrid virions multiplied in green monkey kidney cells in the presence of human adenovirus types 1, 2, and 3, but not 3 and 7, and acquired the capsid of the helper adenovirus. The hybrid can serve as a helper for human adenoviruses. It can apparently induce T antigen of the C8 virus but, in contrast to the latter, does not induce tumors in hamsters.

It has been recently established (8; Rowe, *personal communication*; Altstein and Dodonova, *unpublished data*) that monkey adenoviruses are capable of enhancing the reproduction of human adenoviruses in green monkey kidney tissue culture (GMK). We have demonstrated (Altstein and Dodonova, *unpublished data*) that, on simultaneous infection of GMK cells by human and monkey adenoviruses, the progeny includes phenotypically mixed particles as well as defective hybrid virions which possess some properties of both original viruses. The present paper describes the hybrid of nononcogenic human adenovirus type 2 and oncogenic monkey SA7 adenovirus.

MATERIALS AND METHODS

Tissue culture and media. Monolayer cultures of GMK and of human embryonic kidney (HEK) cells were prepared by a modification of Youngner's method (15). The growth medium consisted of Hanks solution with 0.5% lactalbumin hydrolysate and 5% bovine serum. After infection, the cultures were maintained in medium 199 without serum.

Viruses. Monkey SA7 adenovirus, C8 strain, was isolated from a rectal smear of a *Cercopithecus aethiops* monkey, and was kindly supplied to us by H. Malherbe. Oncogenic properties of this virus have been described (7; Altstein et al., *in press*). The virus stock used was the 12th passage of this strain in GMK cells. The prototype strains of human adenoviruses, types 1, 2, 3, 5, and 7, were received from the Moscow Institute for Viral Preparations, and were grown in HEK cells. Virus stocks were maintained at −20 C. None of the strains studied induced synthesis of immunofluorescent stainable SV40 T antigen in GMK cells, and none contained infectious SV40 virus.

Immune sera. Neutralizing antisera were prepared in rabbits. Antisera to the T antigen of the C8 virus were obtained from hamsters bearing transplanted and primary tumors induced by this virus. Two pools of these sera were used. Both of them reacted specifically in the complement fixation (CF) test with a tumor antigen from transplanted C8 tumors and did not react with extracts of hamster tumors induced by monkey adenoviruses SA7 (B105), SA7 (C626) (Altstein et al., *in press*), or SV40, or with adenovirus group CF antigen or the C8 viral antigen.

Virus assays. The viruses were titrated by the plaque or end point dilution methods, by using GMK cells for C8 adenovirus and HEK cells for human adenoviruses. Both types of cells were used for titration of the hybrid virus. In titrations by the end point dilution method, four test-tube tissue cultures were used for each 10-fold dilution of the virus; virus dilutions were made in medium 199. Final results of titrations were registered at 3 weeks.

Plaque titrations were done in 50-ml flasks (square surface about 20 cm^2) with rubber stoppers. Adsorption of virus (0.2 ml/flask) was carried out for 1 hr at 37 C. The agar overlay consisted of 1.5% agar (Difco) in Earle's solution with 10% boiled skimmed milk, 0.4% sodium bicarbonate, 1% bovine serum, 0.0017% neutral red, penicillin (100 units/ml), and streptomycin (100 units/ml). Final results of titrations were obtained 14 to 18 days after infection.

Neutralization test. Neutralization was carried out by mixing 0.9 ml of a virus-containing material with 0.1 ml of immune serum, followed by incubation for 2 hr at 37 C. In quantitative neutralization tests, 10-fold dilutions of the virus were mixed with a constant dilution of the serum. Final concentration of the serum in the mixture corresponded to 8 to 16 neutralizing units. (The dilution of the serum inhibiting cytopathogenic activity of 100 $TCID_{50}$ of the homologous virus was considered 1 neutralizing unit.) The degree of neutralization (neutralization index) was expressed as the reduction of the number of plaques induced by a serum-treated virus as compared with the untreated control.

Cloning of virus. Flasks with one to three isolated

plaques were selected for cloning experiments; these were usually carried out 14 to 18 days after infection. Agar from the region of the plaque was suspended in 1.0 ml of medium 199, and two or three tubes of tissue culture were inoculated with this suspension. The virus harvest from these cultures were studied as a separate clone.

Detection of T antigen by CF test. GMK cells grown in Povitskaya flasks (250 ml) were infected with virus at a multiplicity of 0.2 to 0.5 plaque-forming unit (PFU)/cell. After the appearance of clear signs of cytopathic effect (CPE; 2+, 20 to 40 hr after infection), the culture fluid was removed and replaced by 2 ml of saline. The cells were destroyed by three cycles of freezing (−70 C) and thawing (37 C). The cell debris was sedimented by centrifugation at 3,000 rev/min for 20 min. The supernatant fluid contained approximately 3% of the cell extract and was used for detection of the T antigen.

The CF test was carried out in a volume of 0.5 ml, with 2 units of complement and with fixation for 18 hr at 4 C. Antigens were titrated against 4 CF units of two pools of anti-T-C8 hamster sera, by using a pool of anti-T-SV40 hamster sera as a control of specificity. Appropriate anticomplementary and standard positive antigen controls were included in each test. Each specimen tested for presence of the T antigen of the C8 virus in CF was examined twice, with similar results.

RESULTS

Origin of the Ad2C8 hybrid virus. Human adenovirus, type 2 (Ad 2), and monkey C8 adenovirus were simultaneously inoculated into GMK cells at a multiplicity of infection of about 0.1 PFU/cell for each virus. Material harvested after complete cell degeneration was passaged three times in GMK cells. For each passage, the culture fluid was used at a dilution of 10^{-1} after treatment with anti-C8 immune serum. Typical adenovirus CPE appeared 3 to 4 days after infection. The material harvested after the third passage was designated as Ad2C8 and was used for the investigations described below.

It should be emphasized that Ad2 virus did not induce CPE in the GMK cells and could not be adapted to those cells during five blind passages. The C8 virus was easily passaged in GMK cells, but CPE did not develop after the in vitro treatment of the infectious material with anti-C8 serum.

Plaque-forming activity of the Ad2C8 virus. During the study of plaque-forming activity of the Ad2C8, C8, and Ad2 viruses, the following results were obtained: (i) Ad2C8 preparations induced plaque formation in both GMK and HEK cells (the titer of virus in HEK cells being 2 to 3 $\log_{10}$ higher than that in GMK cells); (ii) the C8 virus induced plaque formation only in GMK cells; (iii) the Ad2 virus induced plaque formation only in HEK cells. There was no marked difference in the time of appearance and size of the plaques induced by Ad2C8 and Ad2 in HEK cells. In GMK cells, plaques induced by the Ad2C8 appeared at the same time as the C8 plaques, that is, 8 to 9 days after infection.

Plaque-forming activity of Ad2C8 was neutralized by anti-Ad2 but not by anti-C8 serum, both in GMK and in HEK cells (Table 1). Addition of anti-C8 serum to the agar overlay also failed to inhibit plaque formation by Ad2C8 virus.

Figure 1 presents some examples of the dose-response relationships for plaque-forming activity of the Ad2C8 virus in GMK and HEK cells. When GMK cells were used, the dose-response curve was of two-hit character; in HEK cells, it was one-hit. The dose-response curves for plaque-forming activity of C8 virus in GMK cells and of the Ad2 virus in HEK cells were of one-hit character. These data indicate that two types of virus particles are present in the Ad2C8 preparation, and the interaction of both particles is necessary for plaque formation.

Plaque-forming activity of the Ad2C8 virus in GMK cells was enhanced by simultaneous infection with human adenoviruses. A constant dose of human adenovirus (Adl, Ad2, Ad3, Ad5, Ad7) was inoculated into the GMK flask cultures (multiplicity of infection, 0.1 to 3 $TCID_{50}$/cell) simultaneously with dilutions of Ad2C8. Cultures inoculated only with the Ad2C8 virus or only

TABLE 1. *Neutralization of plaque-forming activity of Ad2C8, C8, and Ad2 viruses by immune sera*

Conditions of treatment by antiserum	Antiserum against virus	Neutralization index			
		GMK Cells		HEK Cells	
		Ad2C8	C8	Ad2C8	Ad2
Mixed with virus in vitro	C8	1.0	>15,000	1.4	1.2
	Ad2	>20,000	1.2	>7,000	>8,000
Added into agar overlay	C8	1.0	>600	1.0	1.1
	Ad2	>1,500	1.0	>7,000	>8,000

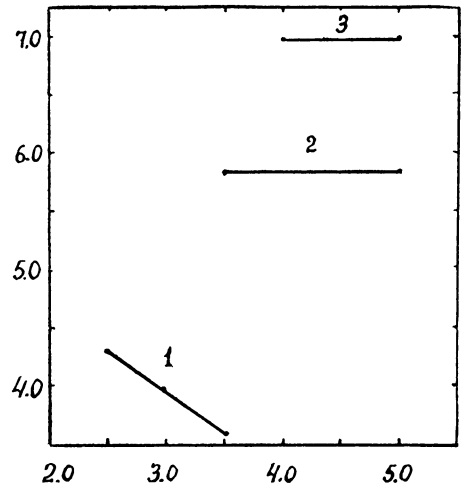

FIG. 1. *Dose-response relation in titrations of Ad2C8 virus by the plaque method in GMK and HEK cells. Abscissa: dilution of Ad2C8 virus. Ordinate: titer estimate (number of plaques × dilution factor)/volume of inoculum. (1, 2) Titration data of Ad2C8 virus in GMK cells without and with addition of helper adenovirus (Ad2), respectively. (3) Titration data of Ad2C8 virus in HEK cells. [This method of plotting dose-response was proposed by Hartley and Rowe (5). A one-hit response curve is horizontal, and a two-hit curve follows a 45° angle.]*

with human adenovirus served as controls. The number of AD2C8 plaques sharply increased in the bottles infected with Ad1, Ad2, or Ad5, but not with Ad3 or Ad7 (Table 2). None of the human adenoviruses tested induced plaques in GMK cells. The dose-response curve for plaque-forming activity of Ad2C8 in GMK cells infected with a lawn of human adenovirus became one-hit (Fig. 1).

Clonal analysis of the Ad2C8 virus. Clones of the Ad2C8 virus from the plaques which appeared in GMK and HEK cells as well as in GMK cells infected with Ad2C8 on a lawn of Ad1, Ad2, or Ad5, were studied. For each clone, the capacity to induce a transmissible CPE in GMK and HEK cells and the capacity to be neutralized with immune sera were investigated.

TABLE 2. *Enhancement of plaque-forming activity of Ad2C8 virus in GMK cells by human adenoviruses*

Helper adenovirus	No. of tests	Multiplicity of infection by helper virus (PFU/cell)	Enhancement of number of plaques formed by Ad2C8[a]
Ad1	8	0.3–3.0	30–110
Ad2	8	0.1–0.6	45–150
Ad3	6	0.5–2.0	0.7–1.1
Ad5	4	0.1–1.0	16–110
Ad7	4	0.1	1.0–1.2

[a] Enhancement was calculated for a 10^{-3} dilution of Ad2C8 virus preparation, and is expressed in terms of multiplicity.

All Ad2C8 GMK clones induced CPE both in GMK and HEK cells and were neutralized by Ad2 serum (Table 3). For these clones, as well as for the uncloned Ad2C8 virus preparations, the plaque-formation dose-response curves were two-hit in GMK cells and one-hit in HEK cells. Ad2C8 HEK clones and Ad2C8 GMK clones passaged once in HEK cells (GMK → HEK) induced CPE only in HEK cells. According to neutralization and cytopathogenic activity, these clones were identified as Ad2.

Table 4 presents data on the Ad2C8 clones from the plaques formed in GMK cells in the presence of added human adenovirus. The clones were neutralized only by the serum homologous to the adenovirus used as helper.

Induction of the C8 T antigen by Ad2C8. Ad2C8 infection of GMK cells induced the T antigen typical of the C8 virus, and T antigen-inducing activity of the Ad2C8 was neutralized by anti-Ad2 but not by anti-C8 serum (Table 5). T antigen-inducing activity of the C8 virus was neutralized by anti-C8 but not by anti-Ad2 serum. The Ad2 virus alone did not induce T antigen reactive with C8 hamster serum.

None of the tested antigens reacted in CF tests with the antiserum against SV40 T antigen, and immunofluorescence tests of GMK cells infected with the Ad2C8, C8, or Ad2 viruses and fixed

TABLE 3. *Properties of Ad2C8 clones*

Clone	Cells used for plating	Cells used for passage of plaque	No. of clones studied	No. of clones cytopathogenic in cells		No. of clones neutralized by sera against	
				GMK	HEK	C8	Ad2
Ad2C8 (GMK)	GMK	GMK	8	8	8	0	8
Ad2C8 (GMK → HEK)	GMK	HEK	8	0	8	0	8
Ad2C8 (HEK)	HEK	HEK	20	0	20	0	20

24 to 48 hr after infection did not reveal T antigen of the SV40 virus.

Oncogenic activity of Ad2C8 virus for hamsters. The C8 virus induced tumors in 10 of 12 newborn hamsters infected subcutaneously with a dose of $10^{3.5}$ PFU/hamster, the latent period being 46 to 75 days. The Ad2C8 virus inoculated into newborn animals ($10^{5.8}$ PFU/hamster) did not induce tumors in 10 infected animals during 360 days after infection.

TABLE 4. *Identification of progeny from enhanced plaques formed by Ad2C8 virus in GMK cells in the presence of helper adenovirus*

Helper adenovirus	No. of clones studied	No. of clones cytopathogenic in cells		No. of clones neutralized by sera against		
		GMK	HEK	Ad1	Ad2	Ad5
Ad1	10	10	10	10	0	0
Ad2	10	10	10	0	10	0
Ad5	8	8	8	0	0	8

DISCUSSION

In mixed infection of cells with human adenoviruses and SV40, adeno-SV40 hybrid viruses appear (6, 10, 12). In our experiments, it was demonstrated that joint infection of GMK cells with human and monkey adenoviruses leads to the formation of hybrids which possess some properties of the original viruses. The hybrid virus preparation induced plaques in GMK cells analogous to those of the C8 virus; in contrast to the latter, the hybrid was neutralized by anti-Ad2 serum. Examination of Ad2C8 preparations by electron microscopy showed typical adenovirus particles; adeno-associated virus particles were not detected (*unpublished data*).

In the study of plaque-forming activity of the

TABLE 5. *Induction of T antigen of C8 virus in GMK cells by C8, Ad2C8, and Ad2 viruses*

T antigen-inducing virus	Hamster serum[a] against T antigen of virus	Results of complement fixation											
		Expt 1						Expt 2					
		Dilution of antigen				Saline	Titer of T antigen	Dilution of antigen				Saline	Titer of T antigen
		1	1:2	1:4	1:8			1	1:2	1:4	1:8		
C8	C8	4+	4+	4+	2+	0	1:4	4+	4+	4+	3+	0	1:8
	SV40	0	0	0	0	0	<1	0	0	0	0	0	<1
C8 + rabbit anti-C8 serum	C8	0	0	0	0	0	<1	0	0	0	0	0	<1
	SV40	0	0	0	0	0	<1	0	0	0	0	0	<1
C8 + rabbit anti-Ad2 serum	C8	4+	4+	3+	0	0	1:4	4+	4+	4+	3+	0	1:8
	SV40	0	0	0	0	0	<1	0	0	0	0	0	<1
Ad2C8	C8	4+	3+	2+	0	0	1:2	4+	3+	1+	0	0	1:2
	SV40	0	0	0	0	0	<1	0	0	0	0	0	<1
Ad2C8 + rabbit anti-C8 serum	C8	4+	4+	1+	0	0	1:2	4+	3+	2+	0	0	1:2
	SV40	0	0	0	0	0	<1	0	0	0	0	0	<1
Ad2C8 + rabbit anti-Ad2 serum	C8	0	0	0	0	0	<1	0	0	0	0	0	<1
	SV40	0	0	0	0	0	<1	0	0	0	0	0	<1
Ad2	C8	0	0	0	0	0	<1	0	0	0	0	0	<1
	SV40	0	0	0	0	0	<1	0	0	0	0	0	<1
None (control)	C8	0	0	0	0	0	<1	0	0	0	0	0	<1
	SV40	0	0	0	0	0	<1	0	0	0	0	0	<1

[a] Both sera were used in dilutions of 1:10. The dilutions were equal to 4 CF units for anti-T C8 serum and 8 CF units for anti-T SV40 serum.

TABLE 6. *Characteristics of the virions of Ad2, C8, and their hybrid Ad2C8*

Virions	Plaque-formation in cells		Neutralization by immune sera against		T antigen induction of specificity		Oncogenicity in hamsters
	GMK	HEK	C8	Ad2	C8	SV40	
Ad2	−	+	−	+	−	−	−
C8	+	−	+	−	+	−	+
Ad2C8 + Ad2	+	+	−	+	+	−	−
Ad2C8[a]	−	−?	−	+	+	−	−

[a] These virions were not physically separated from the mixed (Ad2C8 + Ad2) population.

hybrid virus in GMK cells, it was found that the dose-response curve is of two-hit character. This finding points to the presence of two types of particles, which induce plaque formation only under conditions of joint infection. One of these particles (Ad2) induces plaques in HEK cells independently and can be isolated from the hybrid preparation by cloning in this cell system. The second type of particle could not be isolated from Ad2 by cloning. We designated these particles as the Ad2C8 hybrid virions. Table 6 summarizes some of the properties of these particles.

Hybrid particles are apparently defective; they cannot propagate independently in GMK cells. In simian cells, they grow only in the presence of human adenovirses types 1, 2, and 5, but not of 3 and 7. The capsid of the hybrid virions is similar to that of the adenovirus component; replacement of one helper adenovirus by another results in a change of the capsid of the hybrid virions. Apparently, transcapsidation (9) between the adenovirus and hybrid virions takes place. At the same time, hybrid particles are necessary for the reproduction of the adenovirus component. Apparently, hybrid particles can serve as helpers to human adenoviruses like complete C8 virus (8; Altstein and Dodonova, *unpublished data*).

In contrast to C8 virus, Ad2C8 hybrid particles are apparently nononcogenic (or less oncogenic).

The ability of the hybrid virus preparations to induce T antigen similar to the T antigen of the C8 virus suggests that genetic material of C8 virus is present in the hybrid virions. Apparently, this genetic material provides not only the induction of the specific T antigen synthesis but also complementation with Ad2 in GMK cells as well. It is not clear whether hybrid particles contain the genetic material of the human adenovirus.

Thus, the data obtained on the properties of the Ad2C8 hybrid, and the interpretation of these data, are in general analogous to the data on adeno-SV40 hybrids (3, 9, 11, 13) and on the strain of adenovirus type 7 which contains monkey cell adapting component (MAC; 4). Defective particles of Ad-SV40 are a result of recombination of genetic material of the adenovirus and SV40 (2, 14). The origin of the second type of defective particles (MAC), which appeared during passage of the adeno-virus type 7 in monkey kidney cells, is unknown. Ad2C8 particles appeared under conditions of joint replication of adenoviruses of human and monkey origin, and apparently contain the genetic material of the monkey adenovirus. Study of the mechanism of formation of "adeno-adeno" hybrids is in progress.

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